Investigations on the Toxic & Teratogenic Effects of GRAS Substances on the developing Chicken Embryo (**Oil of Nutmeg**) No Date

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Investigations on the Toxic and Teratogenic Effects of GRAS Substances on the Developing Chick Embryo.[1]

Oil of Nutmeg

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[1]Report of investigations conducted under Contract No. 72-343 with the Food and Drug Administration, PHS, DHEW.

General Protocol:

Ten test substances were supplied by the Food and Drug Administration for testing in the chick embryo. Details on the nature and source of these substances is shown in Table 1. All substances were stored at room temperature in the dark until they were used, except that the propyl gallate and phosphated mono- and di-glycerides were kept under refrigeration. Most of the substances were dissolved in a suitable solvent or suspended in a suitable liquid for injection into fertile eggs. In one instance the substance was injected directly without a solvent or carrier. Specific information about solvents, solubility of the substances and problems peculiar to individual substances will be given under specific protocol for each substance tested.

Fertile eggs used in these investigations were from a specific pathogen free flock of Dekalb 161 egg production type chickens fed a breeder ration free of antibiotics or other drugs. Eggs were stored at 55° F and a relative humidity of 80 percent for 0 to 5 days prior to use. Eggs were allowed to reach room temperature, placed on plastic flats and subjected to ultraviolet irradiation for 30 minutes. The top of each egg was cleansed by a cotton swab saturated with 70 percent ethanol, a small hole was drilled over the air cell through the shell and the test substance was injected with the aid of a 0.25 ml. tuberculin syringe fitted with a suitable needle. All equipment and glassware used to handle the test substances or their solutions or suspensions were sterilized by auto claving and every attempt was made to avoid microbiological contamination of the eggs. Following injection the hole in each egg was sealed by a drop of flexible collodion and the eggs were set in or returned to the incubators. Jamesway Model 252 Incubator-Hatchers were used and maintained at 100° F dry bulb temperature and 86° F wet bulb temperature during the first 18 days of incubation. Eggs were turned automatically each 4 hours. Eggs were candled periodically to remove dead embryos and all embryos were examined for stage of development and obvious defects. After 18 days of incubation viable embryos were transferred to hatching baskets and hatching temperature was reduced to 98.5° F dry bulb reading and humidity was increased to a 90° F wet bulb reading. Upon hatching (22nd day) chicks were examined for abnormalities and samples were cleared and alizarin stained to examine them for skeletal defects. Other embryos (50 for each substance studied) were sacrificed and samples of liver, muscle, bursa, brain, eye, spleen, heart, pancreas, lung and kidney were taken and fixed in formalin. Later tissues were embeded in paraffin, cut, stained and mounted for histopathological examination. Each sample was done in duplicate and hence a total of 10,000 tissues were examined for lesions.

Preliminary range finding experiments were conducted to find the doses of the test substances that could be used in constructing dose response curves for toxicity as measured by embryonic mortality. In two cases, the test substance was non-toxic in the largest dose that could be accommodated by injection. Specific dose response experiments using 100 or more eggs per dose and 5 or more doses of the test substance were conducted at a minimum of 3 time intervals to obtain the toxicity data reported. Solvent or sham injected controls and untreated control groups of eggs were used with each experiment. In some cases, extra trials were conducted to provide embryos for examination at critical doses of the test substances in order to further evaluate teratogenic response and obtain additional data on the nature of embryonic defects.

Data obtained from the experiments (except that from the range finding studies) was transferred to data sheets provided (FDH form 2572, 2572a and 2572b) and submitted to FDA for statistical analysis. Nine types of data summaries including 2 statistical treatments of the data were provided by FDA on the data submitted. The results presented and interpretations made are largely based on these data summaries.

Table i

FDA Project Test Substances

Test Substance and Identification		Compound No.
1. Lactose, Edible Formost Dairies, Inc. Appleton, Wisc.		000063423
2. Propyl Gallate Lot 337		000121799
3. Sodium Ascorbate, U.S.P. FCC Lot No. 965102 Hoffmann-LaRoche Inc., Nutley, N. J. FDA 3167 73(C)		000134032
4. Sodium Erythorbate F.C.C. Lot No. 834072 FDA 3167 73(C) Hoffmann-LaRoche, Nutley, N. J.		977052064
5. Oil Nutmeg NF, East Indian Fritzsche Dodge & Olcott, Inc. 71-28 New York, N. Y.		MX 8008455
6. Zinc Sulfate - Rayon Lot # 2132R1 Virginia Chemicals, Inc. Portsmouth, Va.	Anhyd. Monohyd.	007733020 007446197
7. Stannous Chloride, AR $2H_2O$ Mallinckrodt Chemical Works St. Louis, Mo.		007772998
8. Talc USP #141, Whittaker, Clark and Daniels, Inc.		010101390
9. Carob Bean Gum FDA 71-14		PM 9000402
10. Phosphated Mono- and Di-Glycerides Lot No. 126 Witco Chemical Organics Division New York, N. Y. EMCOL D70-30C		977051323

General Discussion and Comparisons:

A comparison of the relative toxicity of the ten compounds tested is shown in Table ii. When toxicity is evaluated by the air cell route of injection at 96 hrs. of incubation, which was the most sensitive for most of the substances tested, it may be seen that the test substances can be divided into 3 categories of toxicity. Substances highly toxic are zinc sulfate, propyl gallate and carob bean gum. Moderate toxicity was encountered with sodium ascorbate, sodium erythorbate, oil of nutmeg and stannous chloride. Those substances of low toxicity were lactose, talc and phosphated mono- and di-glyceride.

Most of the substances tested produced general embryo toxic response as ascites and/or edema except for lactose and talc at the doses tested. Some specific structural defects were noted and seemed to be related to certain substances as shown in Table ii.

Table ii

Comparison of Ten Substances Tested
for Toxicity and Teratology

Substance Tested	LC_{50} via air cell at 96 hrs.	Specific Abnormalities Noted
Lactose	very large	none
Propyl Gallate	13 mgs./kg.	Ascites, edema, celosomia.
Sodium Ascorbate	100 mgs./kg.	Ascites, edema, celosomia, liver histopathology, head defects.
Sodium Erythorbate	84 mgs./kg.	Ascites, liver histopathology.
Oil of Nutmeg	240 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Zinc Sulfate	4 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Stannous Chloride	120 mgs./kg.	Ascites, edema, celosomia.
Talc	>200 mgs./kg.	none
Carob Bean Gum	23 mgs./kg.	Anophthalmia, phocomelia, micromelia, torticollis, celosomia.
Phosphated Mono- and Di-Glycerides	>3000 mgs./kg.	Ascites, anophthalmia, brachygnathia.

V. OIL OF NUTMEG

Specific Protocol:

Oil of Nutmeg was a liquid at room temperature and found to be toxic in very small volume when administered directly without dilution. Corn oil was used as a diluent and solvent to permit more accurate administration of a test dose and to keep injection volume constant for all dose levels employed. The corn oil was heated to sterilize it, but the oil of nutmeg was not. Solutions were made under asceptic conditions in sterile containers. Seven dose levels of oil of nutmeg were tested both at 0 and 96 hrs. of incubation and via both air cell and yolk routes of administration.

Results:

The data for oil of nutmeg is presented in Tables 17-20. When given at 0 hr. by either air cell or yolk administration high solvent control mortality was observed due to the corn oil carrier. Hence only large doses of (13.2 to 22.0 mgs./egg) oil of nutmeg produced highly significant increases in percent mortality when given at 0 hr. When given at 96 hrs. by either air cell or yolk administration the solvent control mortality was low but the response to oil of nutmeg was similar to that observed at 0 hr. in that only the higher levels produced significant or highly significant increases in percent mortality. In all cases except for yolk injection at 0 hr. the regression of dose on mortality was highly significant. When oil of nutmeg was given at 0 hr. by both routes of administration low doses of this substance tended to reduce embryonic mortality suggesting that oil of nutmeg reduced the toxicity of the corn oil carrier.

Significant increases in percent abnormal chicks hatched were observed in most cases where embryonic mortality was increased significantly. An exception to this statement is that a significant increase in percent abnormal chicks hatched was observed when 2.2 or 4.4 mgs. of oil of nutmeg was given via the yolk at 0 hr. This occurred under conditions where a significant decrease in embryonic mortality was noted.

Percent H-S-L-V abnormalities were significantly increased by the two highest levels of oil of nutmeg but only when given at 0 hr. via the air cell. General edema, ascites, celosomia and dwarfism were the specific abnormalities that appeared to be increased by oil of nutmeg administration.

Discussion:

Oil of Nutmeg produced an embryo toxic effect that was closely related to dose. The dose range over which a mortality response was observed was much narrower than for the compounds previously reported in this study. Hence the toxicity range (from no response to 100 percent mortality) was rather small. Considerable embryo toxic response was also observed due to general edema and ascites. Incidence of dwarfism was clearly larger than for the controls or previously mentioned compounds. The LC_{50} at 96 hrs. via the air cell was about 240 mgs./kg.

OIL OF NUTMEG (MG/KG) IN CORN OIL/6/200 ONE OR MORE ABNORMALITIES
OIL OF NUTMEG (MG/KG) IN CORN OIL/6/200 MORTALITY PCT
PER CENT
12.50
25.00
50.00
62.50
75.00
100.00
40.00
50.00

Lab 4

OIL OF NUTMEG (MG/KG) IN CORN OIL/6/096 ONE OR MORE ABNORMALITIES
OIL OF NUTMEG (MG/KG) IN CORN OIL/6/096 MORTALITY PCT
PER CENT
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00

OIL OF NUTMEG (MG/KG) IN CORN OIL/Y/000 ONE OR MORE ABNORMALITIES
OIL OF NUTMEG (MG/KG) IN CORN OIL/Y/000 MORTALITY PCT
PER CENT
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
MG/KG
50.00

Tab 4

OIL OF NUTMEG (MG/KG) IN CORN OIL/1/2005 ONE OR MORE ABNORMALITIES
OIL OF NUTMEG (MG/KG) IN CORN OIL/1/2005 NORMALITY PLT
PER CENT
12.50
25.00
50.00
75.00
87.50
100.00
50.00

Lab 4

Table 17

DATA SUMMARY

Oil of Nutmeg in Corn Oil
via Air Cell at 0 Hr.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5,5a]	Percent H-S-V-L Abnormalities
Control	None	516	5.18	4.65	0.77
Solvent	None	120	41.66	7.50	0.83
8.8	0.44	79	30.37	5.06	2.53
44.0	2.20	79	24.05[1]	1.26	0
88.0	4.40	119	43.69	8.40	2.52
176.0	8.80	39	43.58	10.25	5.12
264.0	13.20	119	71.42[1a]	20.16[2]	2.52
352.0	17.60	39	84.61[1a]	20.51[2a]	7.69[3a]
440.0	22.0	118	88.98[1a]	24.57[2]	6.77[3a]

[1] Difference from control group is significant

[1a] Difference from control group is highly significant

[2] Difference from control group response is highly significant

[2a] Difference from control group response is significant

[3a] Same as 2a

[4] Regression of dose on mortality is highly significant

LC_{30} = 219 mgs./kg.
LC_{50} = 283 mgs./kg.
LC_{70} = 367 mgs./kg.
LC_{90} = 533 mgs./kg.

[5] Regression of dose on abnormal chicks is significant

[5a] Regression of dose on 2 or more abnormalities is significant

Table 18

DATA SUMMARY

Oil of Nutmeg in Corn Oil
via Air Cell at 96 Hrs.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	516	5.18	4.65	0.77
Solvent	None	138	14.49	8.69	0.72
8.8	0.44	100	11.00	11.00	0
44.0	2.20	99	13.13	9.09	2.02
88.0	4.40	139	15.10	14.38	1.43
176.0	8.80	40	17.50	17.50	5.00[3]
264.0	13.20	140	75.71[1]	33.57[2]	2.14
352.0	17.60	39	100.00[1]	2.56	5.12
440.0	22.0	140	100.00[1]	0[2]	0

[1] Difference from control group is highly significant

[2] Difference from control group response is highly significant

[3] NS

[4] Regression of dose on mortality is highly significant

LC_{30} = 221 mgs./kg.
LC_{50} = 240 mgs./kg.
LC_{70} = 261 mgs./kg.
LC_{90} = 294 mgs./kg.

[5] NS - F (Cal) $<$ F (.05)

Table 19

DATA SUMMARY

Oil of Nutmeg in Corn Oil
via Yolk at 0 Hr.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	516	5.81	4.65	0.77
Solvent	None	120	54.16	2.50	0.83
8.8	0.44	78	41.02[1a]	5.12	1.28
44.0	2.20	80	40.00[1a]	8.75[2a]	3.75
88.0	4.40	119	39.49[1a]	8.40[2a]	0.84
176.0	8.80	40	60.00	7.50	2.5
264.0	13.20	120	88.33[1]	12.50[2]	4.16[3]
352.0	17.60	40	100.00[1]	7.50	0
440.0	22.0	119	100.00[1]	0	0

[1] Difference from control group is highly significant

[1a] Difference from control group is significant

[2] Difference from control group response is highly significant

[2a] Difference from control group response is significant

[3] NS

[4] Too few points on dose curve

[5] NS - F (Cal) $<$ F (.05)

Table 20

DATA SUMMARY

Oil of Nutmeg in Corn Oil
via Yolk at 96 Hrs.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	516	5.81	4.65	0.77
Solvent	None	134	14.92	11.94	3.73
8.8	0.44	99	13.13	5.05	2.02
44.0	2.20	100	17.00	9.00	1.00
88.0	4.40	138	15.94	13.04	4.34
176.0	8.80	40	32.50[1a]	27.50[2a]	0
264.0	13.20	140	47.85[1]	36.42[2]	5.00
352.0	17.60	39	89.74[1]	12.82	5.12[3]
440.0	22.0	139	99.27[1]	5.10[2a]	2.91

[1] Difference from control group is highly significant

[1a] Difference from control group is significant

[2] Difference from control group response is highly significant

[2a] Difference from control group response is significant

[3] NS

[4] Regression of dose on mortality is highly significant

LC_{30} = 251 mgs./kg.
LC_{50} = 279 mgs./kg.
LC_{70} = 310 mgs./kg.
LC_{90} = 361 mgs./kg.

[5] NS - F (Cal) < F (.05)